Polysaccharide Production by a Gram Negative Facultatively Anaerobic Rod

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그람陰性 稈菌에 의한 多糖類의 生產

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A bacterial isolate FRI-33 which produces hydrophillic polysaccharide was identified and its cultural condition was investigated. FRI-33 was identified as *Enterobacter agglomerans*. The optimum cultural conditions for polysaccharide production were 30 °C, pH 5.7, using medium composed of glucose 25 g/l, peptone 2 g/l, yeast extract 0.5 g/l, KH_2PO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 1 g/l, $CaCO_3$ 2.5 g/l. The polysaccharide production after 72 hours was 8.41 g/l. The polysaccharide was composed of galactose (1.0 mole), xylose (1.5 mole), gluconodeltalactone (1.9 mole) and ribose (0.03 mole). The apparent viscosity of 1 % polysaccharide solution was 504 mPa.s at 60 rpm and intrinsic viscosity was 45.80 dl/g.

Polysaccharides are very important materials as stabilizers or gelling agents in pharmaceutical(1,2), paint(5) and food industries(1,2,6). They are produced mainly from seeds, plant exudate and seaweeds(6). The availabilities of the raw materials depend upon many environmental factors(2). In this sense, the microbial production of polysaccharides by fermentation is preferred to the conventional production. Many microorganisms (8-10) synthesize a variety of polysaccharides which have unique and useful rheological properties(11-16). In a series of screening program of polysaccharide-producing bacteria, we isolated several candidates capable of producing a viscosifying polymer. This paper reports the cultural condition of strain FRI-33 and viscometric data of the polysaccharide.

Materials and Methods

Microorganism and cultivation

Strain FRI-33, which was from plant, was used in this study. The test bacterium was grown on Nutrient Agar at 30 °C and transferred every two weeks. Starter culture was grown in YM broth (Difco). The inoculum was 5%. The medium used contained the following composition per liter of distilled water: Glucose 25 g/l, peptone 2.0 g/l, $MgSO_4 \cdot 7H_2O$ 1.0 g/l, yeast extract 0.5 g/l, $CaCO_3$ 2.5 g/l. Culture was performed in 250 ml erlenmeyer flask on rotary shaker (120 rpm) or Bioflo C-30 fermentor (New Brunswick, U.S.A., working volume: 1.5 l, 400 rpm, 1vvm).

Polysaccharide recovery(17,18)

Isopropanol precipitiation technique was used for recovery. Two volumes of isopropanol was mixed with cell-free supernatant of culture broth and the precipitated polysaccharide was filtered. The recovered polysaccharide was freezedried and weighed.

Hydrolysis of polysaccharide

The purified polysaccharide was digested with 2

N sulfuric acid in boiling water bath for 24 hours, neutralized with barium hydroxide, filtered and desalted with Amberlite IR-120 column chromatography.

Analysis(19)

Dry cell weight was determined by turbidometry. Sugar was determined by HPLC (Waters, U.S.A.) with Carbohydrate analysis column and RI detector. The mobile phase was acetonitrile/water (85/15) and the flow rate was 1.5 ml/minutes. Viscosity was measured by Brookfield viscometer (Spindle No. 3, 25°C, Brookfield Inst. Co., U.S.A.) and Cannon Fenske capillary viscometer (U.S.A.).

Identification of microorganism(20)

The microorganism was identified by the method described in Bergey's Manual of Systematic Bacteriology.
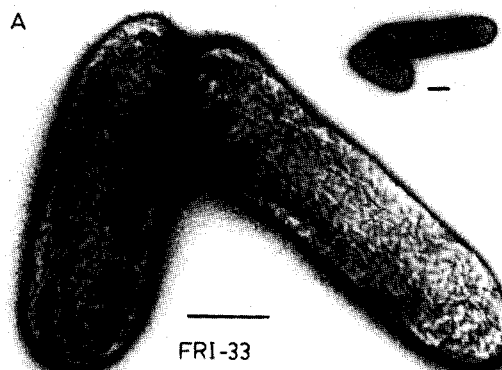
Result and Discussion

Fig. 1. Transmission electron micrograph of strain FRI-33 (A: Higher magnification, Bar = 0.5 μm)

Identification of bacterium

The strain FRI-33 was identified by morphological and biochemical characteristics (Table 1, Fig. 1). The organism was gram negative, facultatively anaerobic, motile rod (0.71-0.98 × 1.48-2.94 μm) with fermentative metabolism. It produced acid and gas from glucose, raffinose, trehalose, xylose, maltose, mannose, rhamnose and arabi-

Table 1. Morphological and biochemical characteristics of strain FRI-33

Reaction	Record	Reaction	Record
Gram reaction :	negative	Acid and gas from	
Colony	: transparent	Glucose	: positive
Motility	: positive	Galactose	: positive
Cell size	: 0.71-0.98 × 1.48-2.94 μm	Raffinose	: positive
Metabolism	: fermentative	Trehalose	: positive
Growth	: facultatively anaerobic	Xylose	: positive
Oxidase	: negative	Maltose	: positive
Catalase	: positive	Lactose	: positive
Urease	: negative	Fructose	: positive
Gelatin hydrolysis :	negative	Sucrose	: positive
Lysine decarboxylase :	negative	Mannose	: positive
Ornithine decarboxylase :	negative	Rhamnose	: positive
Arginine dihydrolase :	positive	Mannitol	: positive
Indole formation :	negative	Ribose	: positive
Hydrogen sulfide(TSI) :	negative	Arabinose	: positive
DNase	: negative	Dulcitol	: positive
Methylred	: negative	Salicin	: positive
VP	: positive	Utilization of	
Nitrate reduction	: positive	Citrate	: positive

nose. It did not utilize malonate but citrate. Positive reactions were recorded in arginine dihydrolase, nitrate reduction and VP tests. Negative reactions were found in lysine decarboxylase, ornithine decarboxylase, methylred, starch hydrolysis, hydrogensulfide and DNase formation. From these characteristics, FRI-33 was identified as *Enterobacter agglomerans*(20).

Cultural condition

Optimum carbon source: To find optimum carbon source for polysaccharide production by *Enterobacter agglomerans* FRI-33, mono-, di- and trisaccharide were used as carbon sources (Table 2). *Enterobacter agglomerans* FRI-33 produced much more cell from raffinose and lactose than other sugars used. The organism preferred glucose and mannose for polysaccharide production and produced 8.42 g/*l* (yield: 33.68 %) and 8.43 g/*l* (yield: 33.72 %) of polysaccharide from the respective sugar. The specific rates of polysaccharide production were 99.11 and 96.76 *mg/g*-cell/*h*. It also produced copious amount of polysaccharide from sucrose and maltose, however, lactose was not satisfactory. The culture viscosity was very high when using raffinose, sucrose and maltose. The apparent viscosities were 820, 556 and 522 mPa.s. It was interesting that culture viscosity was very low on mannose medium where more than 8 g/*l* of polysaccharide was produced. The fact might be due to the possible change in the properties of polysaccharides of the

polysaccharide produced depending upon the carbohydrates used(21). This kind of difference in culture viscosity caused by carbon source was reported by Nakayama *et al.*(22). The pH change was observed on glucose and mannose media. From the above result, glucose was concluded as an optimum carbon source. Normally, glucose(23), fructose(22) and sucrose(17) were major substrates for microbial polysaccharide production.

Optimum nitrogen source: Organic and inorganic nitrogen source was added to the liquid medium at the concentration of 2 g/*l* and compared for the effect on the polysaccharide production (Table 3).

Peptone was found to be the best nitrogen source for the polysaccharide production by *Enterobacter agglomerans* FRI-33 and ammonium sulfate was secondly favorable. The polysaccharide produc-

Table 3. Effect of nitrogen sources on the polysaccharide production by *Enterobacter agglomerans* FRI-33 after 72 hours of incubation at 30°C

Nitrogen source	Crude polymer (g/*l*)	Yield (%)	Final pH
NH$_4$Cl	4.81	19.24	6.27
Urea	3.60	14.40	6.40
(NH$_4$)$_2$SO$_4$	7.40	29.60	6.64
NaNO$_3$	3.46	13.84	6.41
Peptone	8.42	33.68	3.60

Nitrogen source: 2g/*l*, Basal medium: Glucose 25g, Yeast extract 0.5 g, MgSO$_4$·7H$_2$O 1g, KH$_2$PO$_4$ 1g, CaCO$_3$ 2.5g/*l*, Initial pH 5.7

Table 2. Effect of sugar sources on the polysaccharide and cell production by *Enterobacter agglomerans* FRI-33 after 72 hours of incubation at 30°C

Sugar source	Cell(g/*l*)	Crude(g/*l*)polymer	Yield(%)	Qp(mg/g/h)	Viscosity(mPa.s)	Final pH
Glucose	1.18	8.42	33.68	99.11	188	3.6
Raffinose	2.44	4.99	21.39	28.40	820	5.9
Lactose	2.29	3.26	13.73	19.77	42	5.6
Mannose	1.21	8.43	33.72	96.76	18	3.4
Sucrose	1.62	6.06	25.52	51.96	556	5.6
Maltose	1.65	6.00	25.26	50.51	522	5.7
Fructose	1.37	5.24	20.96	53.12	470	5.1

Viscosity was measured by Brookfield viscometer with spindle No. 3 at 60 rpm at 25 °C. Basal medium: Peptone 2g, Yeast extract 0.5g MgSO$_4$·7H$_2$O 1g, KH$_2$PO$_4$ 1g, CaCO$_3$ 2.5 g/*l*, Sugar; Glucose, Mannose Fructose 25g, Raffinose 23.33g, Lactose, Sucrose, Maltose 23.75g, Initial pH 5.7

tions (yields) for the respective nitrogen source were 8.42 g/*l* (33.68 %) and 7.40 g/*l* (29.60 %). Nitrogen source was reported to affect the synthesis of microbial cell(23) and normally extracellular polysaccharide was synthesized in nitrogen-limited culture(22,24).

Optimum pH: Table 4 shows the effect of initial pH on the polysaccharide production by *Enterobacter agglomerans* FRI-33. The polysaccharide production was higher at lower pH and decreased with the increase of initial pH. The optimum pH was concluded to be 5.7 which was similar to those for *Bacillus polymyxa*(25) and *Alcaligenes facalis* (26). Amemura and Harada(27) reported that introduction of buffering system for pH control could not be satisfactory to raise the production yield so much as the addition of calcium carbonate.

Optimum temperatures: Effect of incubation temperature on the polysaccharide production is shown in Table 5. The biomass accumulation was not so much affected by temperature change, however, the optimum temperature for polysaccharide production by *Enterobacter agglomerans* FRI-33 was found to be 30 °C. The polysaccharide production and specific rate of polysaccharide production were 8.41 g/*l* and 98.99 *mg/g*-cell/*h*, respectively. The optimum temperature was similar to those reported by Iwamuro *et al.*(28) and Yim *et al.*(29).

Fermentation pattern

Enterobacter agglomerans FRI-33 was grown in glucose medium in 2 *l* jar fermentor and polysac-

Table 4. Effect of initial pH on the polysaccharide production by *Enterobacter agglomerans* FRI-33 after 72 hours of incubation at 30°C

Initial pH	Crude polymer (g/*l*)	Yield (%)
4.5	7.74	30.95
5.7	8.42	33.68
6.5	4.51	18.04
7.5	4.91	19.62
8.5	4.52	18.08

Medium: Glucose 25g, Peptone 2g, Yeast extract 0.5g, KH$_2$PO$_4$ 1g, MgSO$_4$ 1g, CaCO$_3$ 2.5g/*l*

Table 5. Effect of temperature on the polysaccharide and cell production by *Enterobacter agglomerans* FRI-33 after 72 hours of incubation at 30°C

Temp.(°C)	20	25	30	35	40
Cell (g/*l*)	1.09	1.11	1.18	1.36	1.11
Crude polymer (g/*l*)	7.46	8.20	8.41	8.14	8.14
Yield (%)	29.84	32.80	33.64	32.58	32.54
Qp (mg/g/h)	95.06	102.60	98.99	83.13	101.85
Final pH	3.40	3.60	3.60	3.60	3.70

Medium: Glucose 25g, Peptone 2g, Yeast extract 0.5g, MgSO$_4$·7H$_2$O 1g, KH$_2$PO$_4$ 1g, CaCO$_3$ 2.5 g/*l*, Initial pH 5.7

charide production was monitored (Fig. 2). The glucose was exhausted in the initial stage of fermentation, biomass and polysaccharide production were drastically accumulated. Final biomass and polysaccharide production were 2 g/*l* and 4.7 g/*l*, respectively. pH of culture broth dropped to 3.8. Maximum volumetric glucose consumption rate, volumetric polysaccharide production rate and yield were 1.58 g/*l*/h, 0.36 g/*l*/h and 23.05 %.

Sugar analysis of polysaccharide

Sugar composition of sulfuric acid hydrolyzate of the polysaccharide produced by *Enterobacter agglomerans* FRI-33 was as shown in Fig. 3. The polymer was found to be a heteropolysaccharide composed of galactose (1.0 mole), xylose (1.5 mole), gluconodeltalactone (1.9 mole) and ribose (0.03 mole).
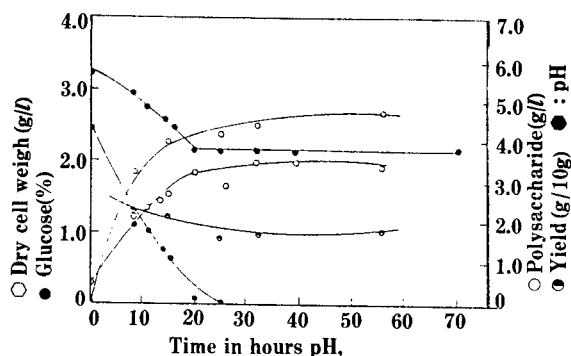

Fig. 2. Time course of polysaccharide production by *Enterobacter agglomerans* FRI-33 at 30°C (air: 1vvm, agitation: 400rpm)
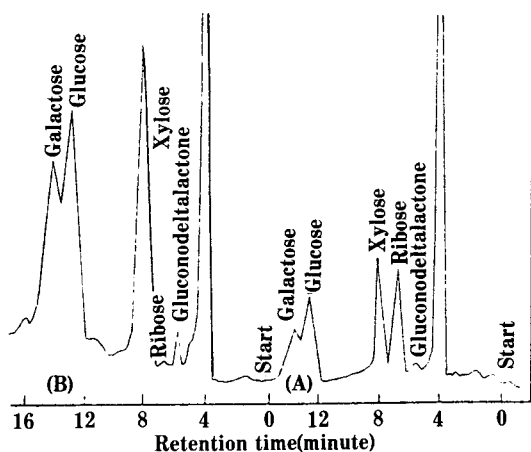
Fig. 3. High performance liquid chromatograms of standard mixture(A) and FRI-33 polysaccharide hydrolyzate(B) Solvent: Acetonitrile/Water (85:15)

Viscometry of polysaccharide solution

Capillary viscometry(30) for dilute solution was conducted to measure the intrinsic viscosity of FRI-33 polysaccharide by fitting to Kraemer's and Huggins' equation (Fig. 4). The intrinsic viscosity was 45.80 dl/g. The relative viscosity of 0.003 % aqueous solution was 1.12. Concentration dependency of aqueous solution was also studied (Fig. 5). The viscosities of 0.5%, 1.0% and 2% solution were 50, 504 and 3,748 mPa.s at 60 rpm. When analyzing the data, double logarithmic plotting was more probable as follow(31,32):

$$\log K = \log K_0 + a \log C$$

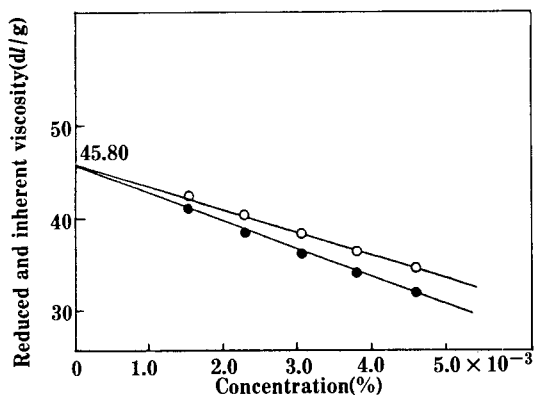where K is viscosity, K_0 is intercept and C is concentration.

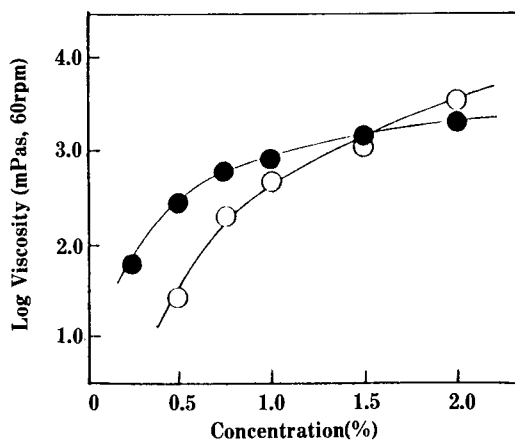**Fig. 4. Reduced and inherent viscosity of FRI-33 polysaccharide (○: RV, ●: IV)**

Fig. 5. Viscosity changes with concentration
●: Xanthan gum, ○: FRI-33 polysaccharide

The a value and K_0 were 2.98 and 432 mPa.s, respectively. Fig. 6 shows the shear rate dependency of 0.25 % FRI-33 polysaccharide solution when fitting to Power law equation. The flow behaviour index and consistecy coefficient were 0.638 and 73.28 mPa.s, respectively. The aqueous solution had also temperature dependency as shown in Fig. 7, so that viscosity increased with lowering the temperature. The activation energy of flow was 8.328 Kcal/mole, indicating higher sensitivity than the polysaccharide solution produced by *Bacillus polymyxa* (33). Fig. 8 shows viscosity change of polymer in buffer solution. The solution had pH optimum at pH 5.6 for maximum viscosity, which was consis-
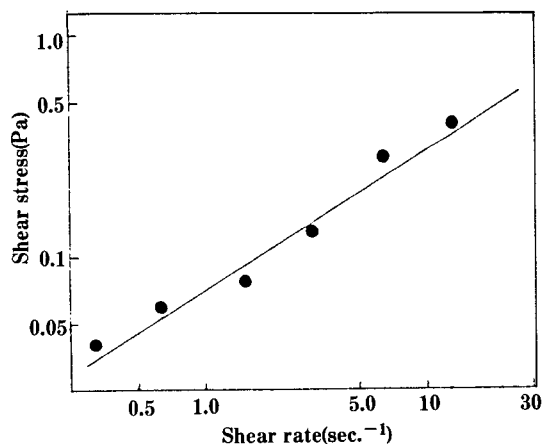

Fig. 6. Shear rate vs. shear stress of FRI-33 polysaccharide solution (0.25%, flow behaviour index: 0.638, Consistency coefficient: 73.28 mPa.s)
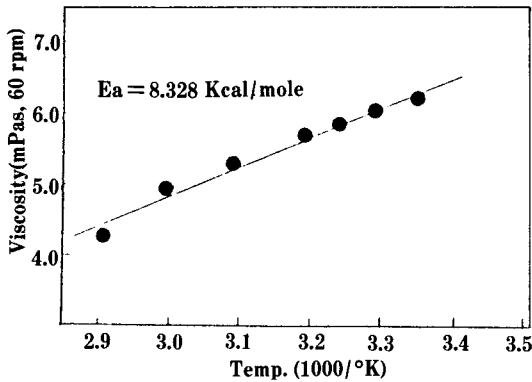
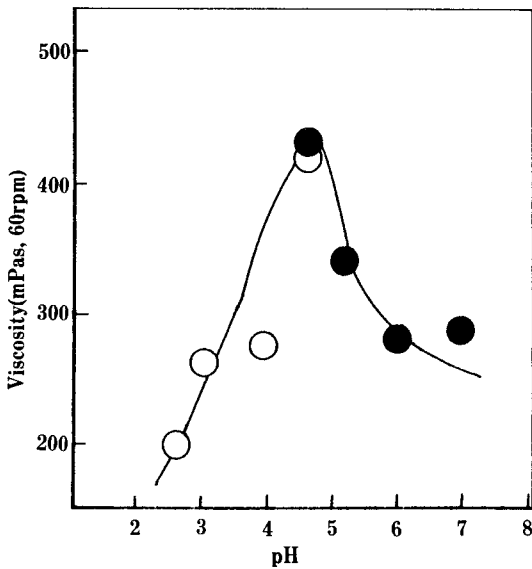**Fig. 7. Arrhenius plot of viscosity of FRI-33 polysaccharide (1.0%)**

Fig. 8. pH dependence of FRI-33 polysaccharide solution (0.98%)

○: Acetate buffer, ●: Phosphate buffer

tent to the optimum pH for polysaccharide production. Some polysaccharides(1) such as xanthangum and PS-7 were reported as having wide range of pH stability.

요 약

微生物 多糖類中 viscosifier를 生產하는 細菌을 分離하고 그 特性을 調査하였다. 本 細菌은 *Enterobacter agglomerans*로 同定되었으며, 多糖類 生產을 위한 條件은 glucose 25 g/*l*, peptone 2.0 g/*l*,

yeast extract 0.5 g/*l*, KH$_2$PO$_4$ 1.0 g/*l*, MgSO$_4$·7H$_2$O 1.0 g/*l*, CaCO$_3$ 2.5 g/*l*, 30℃, pH 5.7이며 이 條件에서의 生產量은 8.41 g/L 이었다. 本 多糖類의 成分은 갈락토오스(1.0몰), 크실로오스(1.5몰), 글루코노델타락톤(1.9몰) 및 리보오스(0.03몰)로 되어 있었다. 1% 溶液의 粘度는 60 rpm에서 504 mpa.s이며 固有 粘度는 45.8 dl/g 이었다.

References

1. Glicksman, M.: *Food Hydrocolloids,* CRC Press, **1,** 127-202 (1982).
2. Blanshard, J.M.V. and Mitchell, R.: *Polysaccharides in Foods,* Butterworths, 251-262 (1969).
3. Warnau, W.C.: US *Patent,* 4,340,678 (1972).
4. Finnerty, W.R. and Singer, M.E.: *Biotechnol.,* **3,** 47-54 (1983).
5. Lindroth, T.A. and Winston, Jr., P.E.: US *Patent,* 4,342,672 (1982).
6. Cheng, H. and Winterdorff, P.: US *Patent,* 4,298,729 (1981).
7. Kang, K.S. and Kovacs, P.: *Proc. Iv. Int. Congress Food Sci. and Technol.,* **1,** 518-522 (1974).
8. Jeanes, A.: *Encyclopedia of Polymer Science* (Mark, H.F., Gaylord, N.G. *eds.*), 8, 693-711 (1968).
9. Yanaki, T., Yamane, T. and Shimizu, S.: *Appl. Microbiol. Biotechnol.,* **23,** 322-329 (1986).
10. Margaritis, A. and Pace, G.W.: *Comprehensive Biotechnol.* (Moo-Young, M. *ed.*), Pergamon Press, **3,** 1006-1044 (1985).
11. Meer, G., Meer, W.A. and Tinker, J.: *Food Technol.,* **29**(11), 22-30 (1975).
12. Higgins, I.J., Best, D.J. and Jones, J.: *Biotechnology,* Blackwell Sci. Pub., 163-212 (1985).
13. Fukui, H., Tanaka, M. and Misaki, A.: *Agric. Biol. Chem.,* **49**(8), 2343-2349 (1985).
14. Holzwarth, G.: *Carbohydrate Res.,* **66,** 173-186 (1978).
15. Bodie, E.A., Schwartz, R.D. and Catena, A.: *Appl. Environ. Microbiol.,* **50**(3), 629-633 (1985).
16. Sandford, P.A.: *Advances in Carbohydrate Chemistry and Biochemistry,* Academic Press, 265-313 (1979).
17. Harada, T., Yoshimura, T., Hidaka, H. and Koreeda, A.: *Agric. Biol. Chem.,* **29**(8), 757-762 (1975).
18. Nakanishi, I., Kimura, K., Suzuki, T., Ishitani, M., Banno, I., Sakane, T. and Harada, T.: *J. Gen. Microbiol.,* **22,** 1-11 (1976).

19. Haibara, T., Amemura, A. and Harada, T.: *J. Ferment. Technol.*, **51**(12), 843-849 (1973).

20. Krieg, N.R. and Holt, J.G.: *Bergey's Manual of Systematic Bacteriology*, Williams & Wilkins, **1**, 408-576 (1984).

21. Kang, K.S. and Cottrell, I.W.: *Microbial Technology* (Peppler, H.J. and Perlman, D. *eds.*), 2nd edition, 417-481 (1979).

22. Nakayama, S., Mitsuda, S., Hirota, T. and Kikuchi, T.: *Hakkokogaku Kaishi,* **5**(1), 31-37 (1979).

23. Williams, A.G. and Wimpenny, J.W.T.: *J. Gen. Microbiol.,* **102**, 13-21 (1977).

24. Duguid, J.P. and Wilkinson, J.F.: *J. Gen. Microbiol.,* **9**, 174-189 (1953).

25. Ninoniya, E. and Kizaki, T.: *Nippon Nogeikagaku Kaishi,* **44**(6), 270-274 (1970).

26. Yamaguchi, M. and Saito, A.: *Rep. Ferment. Res. Inst. (Japan),* **49**, 91-101 (1977).

27. Amemura, A. and Harada, T.: *J. Ferment. Technol.,* **49**(6), 559-564 (1971).

28. Iwamuro, Y., Murata, M., Kanamaru, K., Mikami, Y. and Kisaki, T.: *Agric. Biol. Chem.,* **45**(3), 653-657 (1981).

29. Yim, M.H., Son, H.S., Chung, N.H. and Yang, H.C.: *Kor. J. Appl. Microbiol. Bioeng.,* **12**(3), 219-224 (1984).

30. Carpenter, D.K. and Westerman, L.: *Polymer Molecular weights* (Slade, Jr., P.E. *ed.*), *Techniques and Methods of Polymer Evaluations,* **4**(2), 379-499 (1976).

31. Iwamuro, Y., Aoki, M., Mashiko, K. and Mikami, Y.: *J. Ferment. Technol.,* **61**(5), 505-510 (1983).

32. Speers, R.A. and Tung, M.A.: *J. Food Sci.,* **51**(1), 96-98 (1986).

33. Tako, M.: *Nippon Nogeikagaku Kaishi,* **51**(10), 591-596 (1977).